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         in Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

We hypothesize that **GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue**. To address this hypothesis, we are undertaking a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We report here the results of the first 12 months of this project. The data summarized here indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonism is dose-dependent, transient, and reversible. Furthermore, the results begin to clarify the intracellular pathways involved in the physiology of GHRH in breast cancer cells, implicating P38 kinase and Jun kinase 2, pathways known to be associated with cell proliferation and apoptosis in other tissues. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of tumors. Moreover, the results provide initial information regarding the mechanism of action of endogenous GHRH. Continued study of this system should further illuminate the physiology of GHRH in neoplastic tissue, as well as clarifying the therapeutic potential of GHRH receptor antagonists in human neoplasia.

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INTRODUCTION

Growth hormone-releasing hormone (GHRH), in addition to stimulating the release of growth hormone (GH) from the pituitary, acts as a trophic factor for pituitary somatotrophs[1-8]. In addition, GHRH is expressed outside of the hypothalamic/pituitary axis, with significant expression in the gonads, gastrointestinal tract, pancreas, thymus, and lymphocytes[9-14]. Expression has also been reported in a variety of tumors(15), including pancreas, lung, CNS[16] and, recently, breast, prostate, and endometrium[17-19]. While the role of extrahypothalamic GHRH is unknown, mitogenic effects have been demonstrated on lymphocytes and germ cells *in vitro*[20,21]. The current study examines the hypothesis that ***GHRH functions as an autocrine/paracrine growth factor in neoplastic breast tissue.*** To address this hypothesis, we have undertaken a comprehensive examination of the physiology of GHRH in immortalized breast cancer cell lines. We examine the effect on cell growth and proliferation of exogenous GHRH and disruption of endogenous GHRH with specific inhibitors. We also identify the intracellular signaling pathways that mediate the effects of GHRH on breast cancer cells. Finally, we will dissect the mechanism by which GHRH and its receptor begin to be expressed in the process of neoplastic transformation of breast tissue. We report here the results of the first 12 months of this project. During this period, we have demonstrated that disruption of endogenous GHRH inhibits proliferation and stimulates apoptosis in breast cancer cell` lines and that this effect is mediated through a pathway involving the MAP kinases, ERK ½, p38 and JunK, as well as the caspase cascade.

BODY

Task 1: To determine the relationship between expression of GHRH, expression of GHRH receptor, and cellular proliferation in breast cancer cell lines (Months 1 - 15)

Quantitate the expression of protein and mRNA for GHRH and GHRH receptor in breast cancer cell lines (Months 1 - 4)

The aim of this task was to determine the variability of expression of GHRH and its receptor in various cell lines in order to develop a spectrum of reagents with varying expression of each of the components of the system. While we have determined that MDA231 cells express relatively large amounts of GHRH by western blot hybridization, this technique has not proved sufficiently sensitive to quantitate expression of the peptide by other cell lines. Since experiments demonstrate clear physiologic effects of disruption of endogenous GHRH even in cell lines in which GHRH could not be demonstrated by western blot, it is clear that a technique of greater sensitivity for the measurement of GHRH will be required for lower expressing cell lines. We are currently exploring options to address this need, including competitive binding assays. We are also working to expand the number of cell lines that we are examining for GHRH synthesis.

Similarly, we demonstrated the presence of the mRNA for the GHRH receptor (GHRHr) in a variety of breast cancer cell lines, including MDA231, T47D, and MCF7 cells, information that was included in the original application. There is clearly a spectrum of expression, with high level expression in MDA231 cells, and much lower expression in the other cell types examined. We have undertaken two approaches to quantitating this difference – real-time fluorescent PCR to quantitate mRNA and receptor binding assays to determine receptor protein expression. Once again, we have experienced

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technical difficulties that have slowed progress on this task. Design of tagged primers for real time PCR has required a number of attempts to avoid amplification of related receptor superfamily members and genomic sequences. This work continues and is expected to yield reliable results in the next few months. Similarly, the development of a receptor binding assay to measure the critical feature of cell-surface expression of receptor has been hampered by difficulty in obtaining the fluorescent-tagged GHRH reagent necessary for this work. It is anticipated that this reagent will again become available during the summer of 2001. In the meantime, we are pursuing the alternative of employing a polyclonal anti-GHRH receptor antibody[22] and semi-quantitative imaging available within our Department.

Define the effect of GHRH and its antagonists on the growth, proliferation, and apoptosis of breast cancer cells (Months 4 -10)

We have made extensive progress on this task. The results detailed here were presented at the Annual Meeting of the Endocrine Society in June 2000 and are currently being prepared as a manuscript for submission to Cancer Research.

The aim of this task was to determine the physiologic effect of disruption of normal GHRH secretion and action on the proliferation and survival of breast cancer cell lines. Because of the high level of expression of both GHRH and its receptor in MDA231 cells relative to other cell types so far evaluated, these experiments focus predominately on this cell line.

MDA231 cells, originally obtained from ATCC, were grown to confluency under standard conditions in DMEM supplemented with 10% FCS. Prior to experiments, the cells were lifted with PBS/2%EDTA, plated at 8,000/cm in 96-well, 24-well, or 6-well plates, and allowed to attach overnight in DMEM/2%FCS. The competitive GHRH antagonist [N-acetyl-Tyr1, D-Arg2] fragment 1-29Amide(Sigma)(GHRHa) was dissolved in 2%acetic acid/1%insulin-free BSA to a stock concentration of 1 mM. GHRHa was added in 80μl DMEM and allowed to incubate for 1 hour. Control cells were treated with the same final concentration of vehicle alone. The medium was then brought to 2%FCS and allowed to incubate until the indicated times. At the time of counting, the medium was aspirated, the cells lifted with 50μl trypsin, and resuspended in 150 μl of PBS. Four 0.1μl samples of each well were counted by hemocytometer, with 8 replicates per treatment.

As shown in figure 1, a single treatment of MDA231 cells with 3 μM antagonist resulted in an approximately 25% decrease in cell number after 24 hours. Subsequently, cell numbers increased in parallel with control cells, indicating that the effect is transient and reversible. A second treatment after 24 hours led to an additional 24 hours of inhibition of the increase in cell number (not shown).
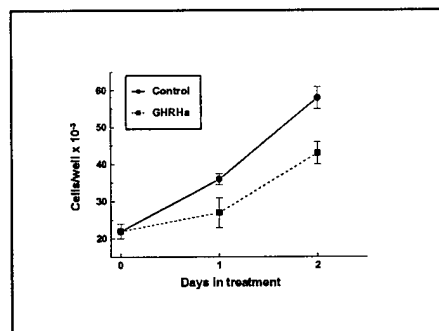

Figure 1: Effect of 3μM GHRHa on MDA231 cell counts *in vitro*
Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment

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The inhibition of cell number increase by GHRHa is also dose dependent. As shown in figure 2, exposure for 24 hours to GHRHa resulted in decreases in cell number ranging from 6% at 40 nM to 25% at 5 μM. Higher doses did not lead to decreases greater than 25%.
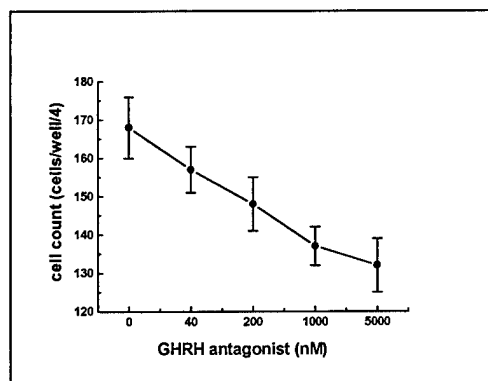

Figure 2: Dose dependent effect of GHRHa on MDA231 cell counts *in vitro*
Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment.

In order to determine whether the effect of disruption of endogenous GHRH on cell number resulted from decreased cell proliferation, we examined the effect of GHRH antagonism on uptake of tritiated thymidine. MDA231 cells were grown in 100□l DMEM/2%FCS in a 96 well plate overnight. GHRHa was added in 80μl DMEM and allowed to incubate for 1 hour. The medium was then brought to 2%FCS. Four hours after GHRHa treatment, 0.8μl ^{3}H-thymidine was added to each well. At the indicated times, cells were washed thrice with 150μl PBS, followed by 25μl 10%TCA. After 5 minutes, 100μl 0.1M NaOH was added followed by 27.5 μl 0.1M HCl. The entire content of the well was transferred to scintillation counter tubes and counted for 10 minutes. Each treatment was examined in 8 replicates.

As shown in figure 3, exposure of MDA231 cells to a single dose of 3 μM GHRHa resulted in a rapid and transient decline in thymidine uptake followed by uptake parallel to control cells. This change in thymidine uptake indicates a decrease in DNA synthesis and suggests a decrease in cellular proliferation. When cells were exposed to a second dose of GHRHa after 4 hours, tritiated thymidine uptake was inhibited for an additional 4 hours followed by recovery and uptake parallel to control cells.
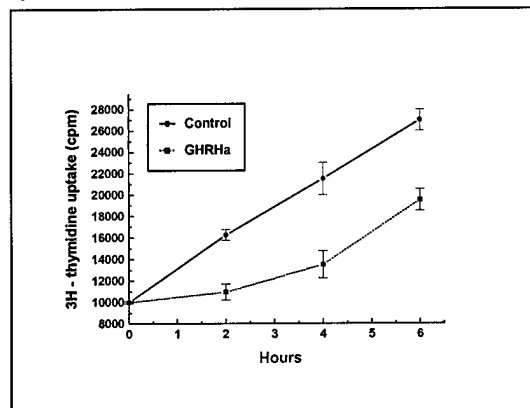
Figure 3: Effect of 3μM GHRHa on ³H-thymidine uptake by MDA231 cells *in vitro*

Values represent the mean ± SEM, n = 8 replicates at each time point for each treatment.

To determine whether antagonism of endogenous GHRH also decreased cell counts through promotion of cellular apoptosis we examined the effect of GHRHa on apoptosis using two independent techniques. First, we examined the effect of GHRHa on DNA laddering. Cells were grown in 3.5cm plates overnight. Following treatment with GHRHa, the volume of medium was increased to 3 ml. Cells were harvested 12 hours after GHRHa treatment, pelleted by centrifugation, and washed once in PBS. Cells were lysed in 20μl lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) and heated to 50C for 1 hour. The mixture was then heated to 90C for 3 min to deactivate the proteinase K, treated with 10 μl RNAse A to a final concentration of 0.5 μg/ml in TE and heated to 50C for 1 hour. Samples were separated by electrophoresis through 2% agarose with ethidium bromide and visualized (Alphaimager).

As shown in figure 4, exposure of MDA231 cells to 3 μM GHRHa lead to a marked increase in DNA laddering compared to vehicle alone, indicating increased apoptosis.
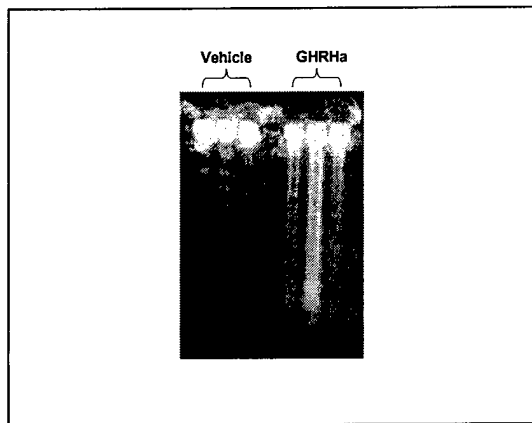
Each lane represents the total DNA sample obtained from a single replicate, 3 replicates per treatment.

Figure 4: Effect of 3 μM GHRHa on DNA laddering in MDA231 cells.

Next, we examined the effect of GHRHa on nuclear condensation, another measure of apoptosis. Cells were grown in 100μl DMEM/2%FCS on chamber slides overnight. GHRHa was added in 80μl DMEM and allowed to incubate for 1 hour. The medium was

then brought to 2%FCS. Twenty-four hours after GHRHa treatment, cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 10 min, followed by 70% EtOH in glycine buffer for 10 min at -20C. Cells were then washed in PBS, incubated with Hoechst dye (8μg/ml) for 15 min at RT, and rinsed three times in PBS. Slides were then blinded and cells were visualized by fluorescent microscopy and apoptotic cells counted (4 fields per slide, eight slides per treatment). (100 X magnification). Exposure of MDA231 cells to 3 μM GHRHa for 24 hours increased the frequency of appearance of condensed nuclei after staining with Hoechst dye. To quantify this increase, we counted the number of condensed nuclei present in a 100X field (4 fields per slide, 8 slides per treatment). As shown in two experiments in figure 5, MDA231 cells exposed to vehicle alone had approximately 30 apoptotic cells per field. However, after 24 hours in the presence of 3 μM GHRHa, the frequency of apoptotic cells increased by 60%.
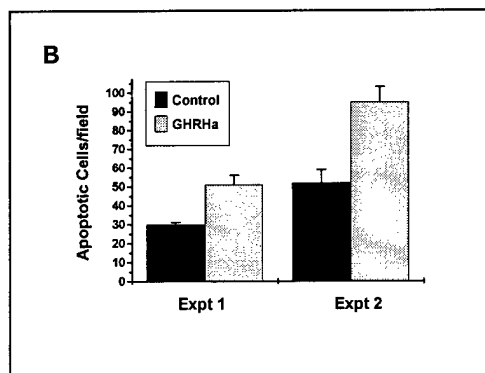

Figure 6: Effect of 3 μM GHRHa on nuclear condensation of MDA231 cells *in vitro*. Each value represents the mean ± SD, n = 8 replicates per treatment, 4 fields counted per replicate.

Exposure of MDA231 cells to GHRHa for 24 hours leads to an approximately 60% increase in the incidence of apoptotic MDA231 cells. However, the total incidence of apoptosis appears to be relatively small, consistent with the previously demonstrated 25% decrease in cell number at 24 hours.

Finally, cells were plated as described, and exposed to GHRH antagonist (3 μM) or vehicle for 24 hours. Cells were then harvested, stained with Krishan's stain, and separated by fluorescent cell sorting. As shown in figure 7, exposure of MDA231 cells to GHRH antagonist results in a marked increase in cells demonstrating apoptotic nuclear signals.
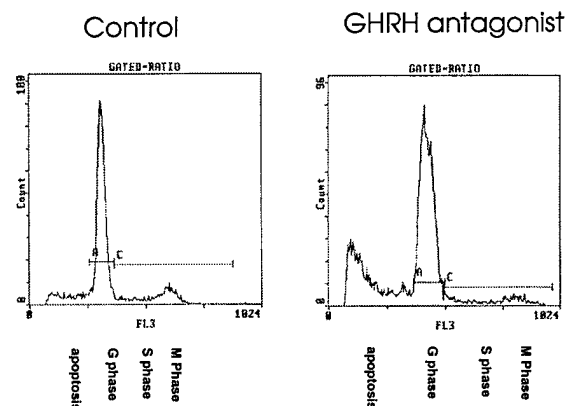
Figure 7: Effect of 3 μM GHRH antagonist on apoptosis determined by FACS analysis in MDA231 cells.

In order to confirm the specificity of these effects of GHRHa, we also examined the effect of additional peptide GHRH antagonists of entirely different structure obtained from Dr. David Coy at Tulane University, peptides PRL 2194 and PRL2640. These peptides have greater affinity for the GHRH receptor than GHRHa. Lyophilized peptides were dissolved in water and added to media at the concentrations shown using an experimental paradigm identical to that used for GHRHa. As seen in figure 8, both of these antagonists lead to a marked dose-dependent decrease in cell number 24 hours after treatment. Furthermore, these peptides with greater affinity for the GHRH receptor than GHRHa have an efficacy that is also greater than GHRHa, leading to cell reductions of up to 65%, compared to 15-25%. The observation that these alternative antagonists, with a structure different from GHRHa reduce cell proliferation and that the degree of reduction correlates crudely with binding affinity further support the conclusion that disruption of endogenous GHRH action impairs normal breast cell proliferation and this effect is mediated through the GHRH receptor itself.
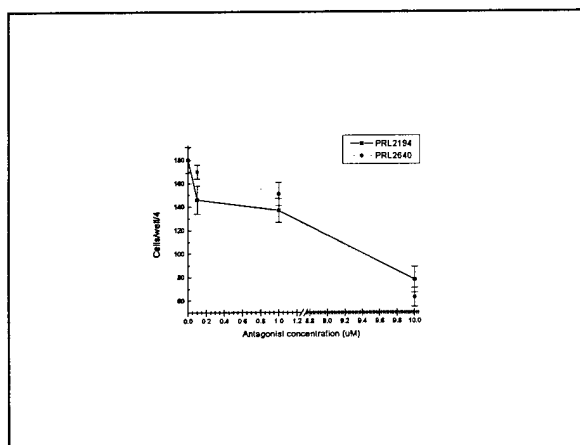

Figure 8: The does dependent effect of peptide GHRH antagonists, PRL2194 and PRL 2640 on MDA231 cell counts in vitro. Each value represents the mean $\pm$ SD, n = 8 replicates per treatment.

Taken together, the data produced as part of this project during the last 12 months indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonists are dose-dependent, transient, and reversible. In addition, the data imply that the effect of GHRH antagonists are mediated through GHRHr itself, rather than cross reaction at related receptors. These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors[23-25] and extend these observations to provide information regarding the mechanism of the effect of GHRH antagonists. The results

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also confirm that breast cell lines *in vitro* provide a useful model system in which to further investigate the physiology of GHRH in breast tumors.

Determine whether changing the expression of GHRH and/or GHRH receptor in breast cancer cell lines results in changes in cell growth characteristics (Months 10-15).

We have obtained the necessary plasmid reagents for this task and will undertake the proposed transfections over the next 4-6 months

Task 2: To determine the intracellular signaling pathway(s) that mediate the effects of GHRH on growth in breast cancer cells (Months 15 - 20)

Define the generation of cAMP and activated MAP kinase pathway intermediates in breast cancer cells in response to GHRH (Months 15-18)
Determine the pathway(s) that mediates the proliferative and/or apoptotic effects of GHRH (Months 19-24)

The work proposed in this task has been started early because of promising preliminary data and the greater availability of reagents relative to the tasks discussed above that have been delayed.

We have previously demonstrated that GHRH, in addition to stimulating the intracellular synthesis of cAMP, also activates the MAPK (ERK ½) pathway in pituitary cells(26). Until this demonstration, it had been assumed that the proliferative actions of GHRH on pituitary somatotroph (GH-secreting cell) were mediated by cAMP generation, as are the hormone releasing actions. However, cAMP stimulation of proliferation is uncommon, since cAMP is much more commonly associated with suppression of cell proliferation(27). Therefore, the identification of involvement of the MAP kinase pathway in GHRH action, a pathway well known to be associated with cellular proliferation, has helped to clarify the mechanism of GHRH effects on cell proliferation.

Therefore, we examined the effect of GHRH on components of the MAPK pathway. Initial experiments focused on the effects of treating MDA231 cells with GHRH. However, it became quickly clear that basal levels of ERK 1/2 activation were significant, likely reflecting the actions of endogenous GHRH. Attempts to decrease basal MAPK activation by manipulation of culture conditions were unsuccessful. It was possible to demonstrate a modest decrease in ERK 1/2 activation in the presence of GHRHa. However, the results were less than compelling.

Since we had demonstrated that antagonism of GHRH leads to increased apoptosis, we turned our attention to other components of the MAPK pathway known to be related to this phenomenon[28-30]. Since activation of these components increases under

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conditions that promote apoptosis, we thought it likely that demonstration of these positive changes would be more straightforward than documenting the negative changes of ERK1/2. This has proven to be the case.

MDA231 cells were exposed to GHRH antagonist (3 μM) or vehicle for 20 min. Cells were then washed, lysed, and the proteins separated by PAGE and analyzed by Western blot hybridization with phospho-specific Jun kinase or Phospho-specific p38 kinase antibody. As shown in figure 9, exposure to GHRH antagonist resulted in marked activation/phosphorylation of both JunK and p38 kinase compared to vehicle.
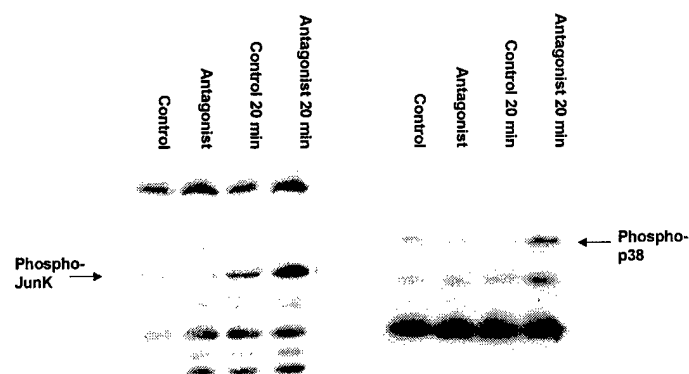

Figure 9: Effect of 3 μM GHRH antagonist on phosphorylation of Jun kinase (left panel) and p38 kinase (right panel) in MDA231 cells.

Activation of the p38 pathway has been associated with initiation of apoptosis in a number of cell systems, while activation of JunK has a more complicated relationship to apoptosis, associated with both stimulation and suppression of apoptosis[28-30].

We next examined the time course of P38 activation in response to GHRHa, using the same experimental paradigm. As shown in figure 10, exposure of MDA231 cells to 3 μM GHRHa leads to dramatic, rapid, and transient phosphorylation/activation of P38, with onset within 20 minutes (Figure 9) and return to baseline before 2 hours. This pattern of P38 activation has been seen in other apoptotic systems.
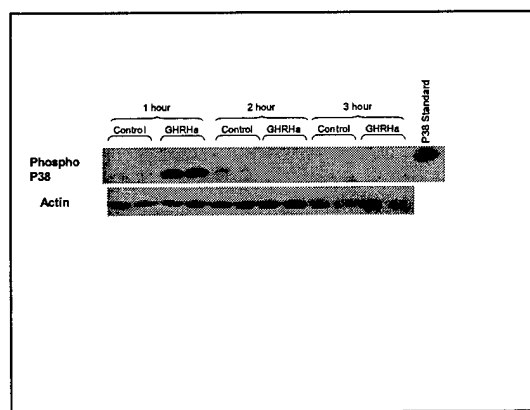

Figure 10: Time course of the effect of 3 μM GHRH antagonist on phosphorylation of p38 kinase in MDA231 cells

In order to determine whether activation of P38 by GHRHa is related to apopotosis, we examined the effect of the P38 inhibitor SB203580 on cell number reduction and apopotosis in response to treatment with GHRHa. MDA231 cells were pretreated with 10 μM SB203580 30 minutes prior to exposure to GHRHa and cell counting, as described above. As shown in figure 11, inhibition of P38 activation by the SB compound prevented the decrease in cell number seen in response to GHRHa, strongly suggesting that activation of P38 mediates the effect of GHRHa on changes in cell number.
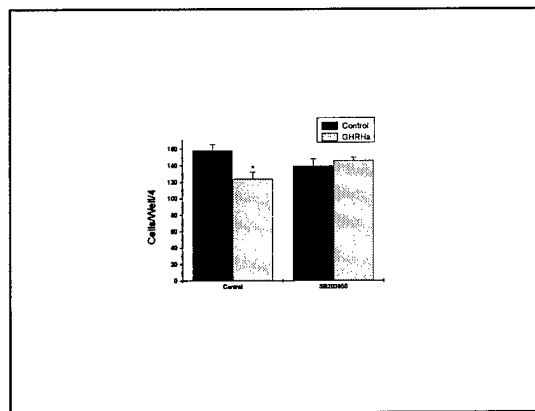

Figure 11: Effect of P38 inhibition on the GHRHa induced decrease in MDA231 cell number.
*Each value represents the mean ± SD, n = 8 replicates per treatment.   * = P<0.05*

We next examined the effect of P38 inhibition on DNA laddering in response to GHRHa treatment of MDA231 cells. Again, cells were pretreated with SB203580 30 minutes prior to exposure to GHRHa and cells processed for DNA laddering as described above. As seen in Figure 12, inhibition of P38 activation by the SB compound prevented the stimulation of DNA laddering seen in response to GHRHa, suggesting that P38 activation is required for the GHRHa stimulation of apopotosis.
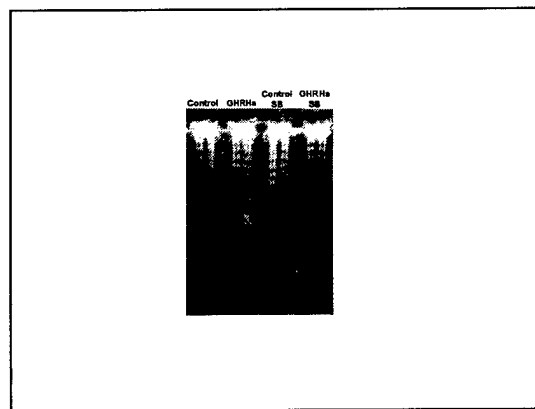

Figure 12: Effect of P38 inhibition on the GHRHa induced apoptosis in MDA231 cells

As noted above, Jun Kinase has a more complicated relationship to apoptosis, being associated with both stimulatory and inhibitory actions under different circumstances. We next examined the effect of inhibition of Jun Kinase on the effect of GHRHa on cell numbers in MDA231 cells. MDA231 cells were transfected with 5 μg of a vector

containing a CMV promoter-driven dominant-negative Jun Kinase 1 (Jnk1 APF) or Jun Kinase 2 (Jnk2 APF) construct by electroporation, grown for 24 hours in serum containing medium and then treated with GHRHa as described above. As seen in figure 14, exposure of MDA231 cells to GHRHa in this experiment resulted in a modest (14%) decrease in cell number after 24 hours. Transfection with dominant negative JnK 1 had no effect on this response to GHRHa. However, transfection with dominant negative JnK 2 more than doubled the decrease (39%). Pretreatment of cells with SB203580 completely prevented the decrease in cell number following exposure to GHRHa, even in cells transfected with dominant-negative Jnk 2 (not shown). The amplification of the effect of GHRHa when JnK 2 is inhibited suggests that activation of JnK 2 antagonizes the effect of P38 activation on cell number.
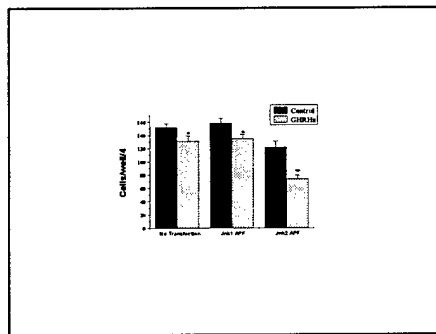

Figure 14: Effect of Jun Kinase 1 and Jun Kinase 2 inhibition on the GHRHa induced decrease in MDA231 cell number.
*Each value represents the mean $\pm$ SD, n = 8 replicates per treatment. * = P<0.05, ** = P<0.01*

This experiment suggests that disruption of endogenous GHRH action on MDA231 cells activates both P38 and JunK 2, the former stimulating and the latter inhibiting apoptosis. It is intriguing to hypothesize that the physiologic state of the cell at the time of GHRH disruption influences the relative degree of activation of these two pathways, leading to alterations in the eventual response of cell proliferation and apoptosis. We are currently exploring this relationship further.

Taken together, the experiments performed to address this task indicate that disruption of endogenous GHRH action on MDA231 cells leads to activation of P38 and Jnk2, along with inhibition of ERK ½. In addition, the effects of GHRH disruption on both cell number and apoptosis are mediated through activation of P38, with Jnk2 perhaps playing a modulatory role. Thus, we can conclude that endogenous GHRH functions in MDA231 cells to stimulate ERK ½ and inhibit activation of P38, thereby promoting cell proliferation and limiting apoptosis. Further examination of both upstream and downstream components of the P38 pathway. Preliminary results indicate that levels of activated caspase 3 are increased 3 hours after treatment with GHRHa, though the exact relationship between activation of P38 and caspase 3 appears to be somewhat complicated. Further studies are currently underway.

Task 3: To define the sequences of GHRH and GHRH receptor (GHRHr) promoters responsible for expression of these genes in breast cancer cells. (Months 24-36)

No work has begun on this task.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that disruption of endogenous GHRH action in MDA231 breast cancer cells with the GHRH antagonist, GHRHa, results in:
  - transient, reversible, and dose-dependent decrease in cell proliferation
  - transient and reversible decrease in thymidine kinase uptake, indicating decreased cellular mitosis
  - increased cellular apoptosis

- Demonstration that disruption of endogenous GHRH action in MDA231 breast cancer cells with the GHRH antagonist, GHRHa, results in:
  - decreased activation of ERK1/2 kinases
  - activation of P38 kinase
  - activation of Jun kinase
  - activation of caspase 3

- Demonstration that inhibition of GHRHa activation of P38 kinase results in:
  - prevention of GHRHa effect on MDA231 cell number
  - prevention of GHRHa-induced apoptosis in MDA231 cells

- Demonstration that inhibition of GHRHa activation of Jun kinase 2 results in amplification of the effect of GHRHa on MDA231 cell number

REPORTABLE OUTCOMES

Zeitler PS, Siriwardana G. Growth hormone releasing hormone (GHRH) stimulates proliferation and inhibits apoptosis in breast cancer cell lines. Abstract presented at the 82nd Annual Meeting of the Endocrine Society, Toronto, Ont, June 200.

Zeitler P, Siriwardana G. Antagonism of endogenous growth hormone-releasing hormone leads to reduced proliferation and apoptosis in MDA 231 breast cancer cells. Manuscript to be submitted to Cancer Resesarch, Summer 2001.

CONCLUSIONS

The data presented here indicate that antagonism of the endogenous GHRH autocrine/paracrine system in MDA231 breast cancer cells leads to inhibition of cell proliferation, as well as increased cellular apoptosis, the combination of which leads to decreased cell number. The effect of GHRH antagonism is dose-dependent, transient, and reversible. Furthermore, the results begin to clarify the intracellular pathways involved in the physiology of GHRH in breast cancer cells, implicating a role for P38

kinase and Jun kinase 2, pathways known to be associated with control of cell proliferation in other tissues types.

These results are consistent with previous demonstrations of an inhibitory effect of GHRH antagonists on a variety of reproductive and GI tract tumors. More importantly, these results significantly extend previous observations by providing initial information regarding the mechanism of the effect of GHRH antagonists and, by implication, the mechanism of action of endogenous GHRH. Furthermore, these results verify the usefulness of breast cancer cell lines as a model system amenable to further molecular dissection of this phenomenon.

It remains unclear at what point during the development of neoplasia GHRH and its receptor appear in breast tissue. Furthermore, the relative importance of this system in the promotion and maintenance of neoplastic changes is unknown. However, continued study of this system will further illuminate the physiology of GHRH in neoplastic tissue, as well as possibly clarifying the therapeutic potential of GHRH receptor antagonists in human neoplasia.

REFERENCES

(1) Li H, Zeitler PS, Valerius MT, Small K, Potter SS. Gsh-1, an orphan Hox gene, is required for normal pituitary development. EMBO Journal 1996; 15:714-724.

(2) Zeitler P, Downs TR, Frohman LA. Development of pituitary cell types in the spontaneous dwarf (dw) rat: evidence for an isolated defect in somatotroph differentiation. Endocrine 1994; 2:729-733.

(3) Asa SL, Kovacs K, Stefaneanu L, Horvath E, Billestrup N, Gonzalez-Manchon C et al. Pituitary adenomas in mice transgenic for growth hormone-releasing hormone. endo 1992; 131:2083-2089.

(4) Barinaga M, Bilezikjian LM, Vale WW, Rosenfeld MG, Evans RM. Independent effects of growth hormone-releasing factor on growth hormone release and gene transcription. Nature 1985; 314:279-281.

(5) Billestrup N, Swanson LW, Vale W. Growth hormone-releasing factor stimulates proliferation of somatotrophs in vitro. Proc Natl Acad Sci USA 1986; 83:6854-6857.

(6) Billestrup N, Mitchell RL, Vale W, Verma IM. Growth hormone-releasing factor induces c-fos expression in cultured primary pituitary cells. Mol Endocrinol 1987; 1:300-305.

(7) Cella SG, Locatelli V, Mennini T, Zanini A, Bendotti C, Forloni GL et al. Deprivation of growth hormone-releasing hormone early in the rat's neonatal life permanently affects somatotropic function. Endocrinology 1990; 127:1625-1634.

(8) Struthers RS, Vale WW, Arias C, Sawchenko PE, Montminy MR. Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. Nature 1991; 350:622-624.

(9) Gonzalez-Crespo S, Boronat A. Expression of rat growth hormone-releasing hormone gene in placenta is directed by an alternative promoter. Proceedings of the National Academy of Science of the USA 1991; 88:8749-8753.

(10) Berry SA, Srivastava CH, Rubin LR, Phipps WR, Pescovitz OH. Growth hormone-releasing hormone-like messenger ribonucleic acid and immunoreactive peptide are present in human testis and placenta. J Clin Endocrinol Metab 1992; 75:281-284.

(11) Bruhn TO, Mason RT, Vale WW. Presence of growth hormone-releasing factor in the rat duodenum. Endocrinology 1985; 117:1710-1712.

(12) Matsubara S, Sato M, Mizobuchi M, Niimi M, Takahara J. Differential gene expression of growth hormone (GH)-releasing hormone (GRH) and GRH receptor in various rat tissues. Endocrinology 1997; 136:4147-4150.

(13) Guarcello V, Weigent DA, Blalock JE. Growth hormone releasing hormone receptors on thymocytes and splenocytes from rats. Cell Immunol 1991; 136:291-302.

(14) Untergasser G, Kranewitter W, Schwarzler P, Madersbacher S, Dirnhofer S, Berger P. Organ-specific expression pattern of the human growth hormone/placental lactogen gene-cluster in the testis. Mol Cell Endocrinol 1997; 130:53-60.

(15) Frohman LA, Szabo M. Ectopic production of growth hormone-releasing factor by carcinoid and pancreatic islet tumors associated with acromegaly. Prog Clin Biol Res 1981; 74:259-271.

(16) Sano T, Asa SL, Kovacs K. Growth hormone-releasing hormone-producing tumors: clinical, biochemical, and morphological manifestations. Endocr Rev 1988; 9:357-373.

(17) Benelot C, Levy L, Fontanaud P, Roche A, rouannet P, Joubert D. Somatostatin and growth hormone-releasing hormone in normal and tumoral human breast tissue: endogenous content, *in vitro* pulsatile release, and regulation. Journal of Clinical Endocrinology and Metabolism 1997; 82:690-696.

(18) Ciocca DR, Puy LA, Fasoli LC, Tello O, Aznar JC, Gago FE et al. Corticotropin-releasing hormone, luteinizing hormone-releasing hormone, growth hormone-releasing hormone, and somatostatin-like immunoreactivities in biopsies from breast cancer patients. Breast Cancer Res Treat 1990; 15:175-184.

(19) Kahan Z, Arencibia JM, Csernus VJ, Groot K, Kineman RD, Robinson WR et al. Expression of growth hormone-releasing hormone (GHRH) messenger ribonucleic acid and the presence of biologically active GHRH in human breast, endometrial, and ovarian cancers. J Clin Endocrinol Metab 1999; 84:582-589.

(20) Fukushima R, Hato F, Kinoshita Y, Tsuji Y, Kitano A. Effect of exogenous growth hormone on in vitro proliferation of thymic lymphocytes from the hypophysectomized rats. Cell Mol Biol 1997; 43:567-579.

(21) Srivastava CH, Breyer PR, Rothrock JK, Peredo MJ, Pescovitz OH. A new target for growth hormone-releasing hormone action in the rat: the sertoli cell. Endocrinology 1993: 133:1478-1481.

(22) Boulanger L, Andersen PH, Gaudreau P. Development of a site-directed polyclonal antibody against the pituitary growth hormone-releasing hormone receptor and its use to estimate GHRH receptor concentration in normal and hypothyroid rats. Neuroendocrinology 1999; 70:117-127.

(23) Csernus V, Schally AV, Kiaris H, Armatis P. Inhibition of growth, production of insulin-like growth factor-II (IGF-II), and expression of IGF-II mRNA of human

cancer cell lines by antagonistic analogs of growth hormone-releasing hormone in vitro. Proc Natl Acad Sci USA 1999; 96:3098-3103.

(24) Kiaris H, Schally AV. Decrease in telomerase activity in U-87MG human glioblastomas after treatment with an antagonist of growth hormone-releasing hormone. Proc Natl Acad Sci USA 1999; 96:226-231.

(25) Kineman RD. Antitumorigenic actions of growth hormone-releasing hormone antagonists. Proc Natl Acad Sci USA 2000; 97:532-534.

(26) Zeitler P, Siriwardana G. Stimulation of mitogen-activated protein kinase pathway in rat somatotrophs by growth hormone-releasing hormone. Endocrine 2000; 12:257-264.

(27) Dumont JE, Jauniaux J-C, Roger PP. The cyclic AMP-mediated stimulation of cell proliferation. Trends Biochem Sci 1997.

(28) Nebreda AR, Porras A. p38 MAP kinases: beyond the stress response. Trends Biochem Sci 2000; 25:257-260.

(29) Harper SJ, LoGrasso P. Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. Cell Signal 2001; 13:299-310.

(30) Ono J, Han J. The p38 signal transduction pathway: activation and function. Cell Signal 2000; 12:1-13.